

TECHNICAL FIELD

PLANT GENE

The present invention relates to methods and materials for
controlling flowering time based on manipulation of
appropriate genes.

PRIOR ART

The timing of flowering in plants is of commercial interest
for a number of reasons. Particularly in those plants in
which the leaves or tubers are a commercial product, it is
desirable to avoid "bolting" (initiation of flowers and stem
elongation) at too early a stage. Conversely it may be
desirable to accelerate flowering under certain circumstances
e.g. to vary flower production across the seasons.
Horticultural plants whose flowering may be controlled
include lettuce, endive and vegetable brassicas including
cabbage, broccoli and cauliflower, and carnations and
geraniums.

It is known that flowering in plants may be dependent on a
number of external factors, which include photoperiodic
control and exposure to low temperatures. Certain plant
hormones and metabolites are known to be able to affect
flowering time. However the underlying mechanisms and
factors controlling the system are not fully understood.

Several studies of flowering have been carried out in
Arabidopsis. It has been shown that once the transition to
flowering happens in *Arabidopsis*, reversion to the vegetative
state rarely occurs. The cells of the apical meristem are
thus-considered stably determined for reproductive
development. Mutagenesis experiments have shown that the
transition and commitment to flowering in *Arabidopsis* is
controlled by many loci (Koornneef et al., 1998).

One locus-*FRIGIDA* (*FRI*), is responsible for the major variation in flowering time in natural ecotypes of *Arabidopsis* (Napp-Zinn, 1987; Lee et al., 1993; Burn et al., 1993; Clarke and Dean, 1994; Sanda and Amasino, 1996). Late flowering is conferred by dominant alleles at the *FRI* locus in all crosses examined so far. The late flowering is very extreme with plants producing around 80 rosette leaves before starting to flower. This should be compared with 7 rosette leaves in the early flowering ecotypes and about 25 in the late flowering mutants at other loci. The extreme phenotype of the late flowering ecotypes can be reversed by a vernalization treatment where the imbibed seeds or plants are incubated at low temperature (below 10°C) before growth at normal temperature (20°C) (Napp-Zinn, 1965). Although the *FRI* locus was not identified among the well characterized late flowering mutants in the Landsberg erecta (Ler) ecotype, recent work has shown that this is due to recessive Ler alleles at a locus on chromosome 5 termed *FLC*. Dominant alleles at *FLC* are required for the late flowering phenotype conferred by *FRI* to be manifested (Lee et al., 1994; Koornneef et al., 1994).

It is likely that *FRI* and *FLC* also play a major role in determining whether Brassica plants require vernalization to flower. Markers linked to the *FRI* and *FLC* have been shown to co-segregate with the two QTL's conferring vernalization requirement in Brassica species (Osborn et al., 1997).

DISCLOSURE OF THE INVENTION

The present inventors have isolated a gene from the *FRI* locus with a demonstrated effect in altering flowering times. The gene shows only very little similarity with known sequences, but appears to encode an alpha helical protein with no membrane spanning domains. In various aspects of the invention the gene, or its variants, may be employed in

manipulating the flowering time trait in a variety of plants.

According to a first aspect of the present invention there is provided an isolated nucleic acid molecule from the FRI locus of a plant encoding a polypeptide which is capable of altering the flowering time of a plant into which the nucleic acid is introduced.

The alteration in flowering time (which may produce a correspondingly altered vegetative phase) may be assessed by comparison with a plant in which the nucleic acid is not present. It may be preferable to use a sample of plants in each case. Flowering time may be measured directly, or inferred from other factors e.g. significant change in leaf numbers at flowering (see Koornneef et al, 1991).

Apart from a quantitative change in flowering characteristics (with associated alteration in leaf numbers etc.) it is preferred that characteristics unrelated to flowering in the plant are substantially unchanged by the polypeptide, which is to say that the polypeptide acts specifically on the flowering characteristic.

Preferably the polypeptide is capable of delaying flowering time (=extending a vegetative phase).

Nucleic acid according to the present invention may include cDNA, RNA, genomic DNA and modified nucleic acids or nucleic acid analogs (e.g. peptide nucleic acid). Where a DNA sequence is specified, e.g. with reference to a figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed.

Likewise, as DNA is generally found in double-stranded form, the complement of those sequences below is also included.

Nucleic acid molecules according to the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or

free or substantially free of other nucleic acids of the species of origin. Where used herein, the term "isolated" encompasses all of these possibilities. The nucleic acid molecules may be wholly or partially synthetic. In particular they may be recombinant in that nucleic acid sequences which are not found together in nature (do not run contiguously) have been ligated or otherwise combined artificially. Alternatively they may have been synthesised directly e.g. using an automated synthesiser.

Most preferably the nucleic acid is derived from the FRI locus of *Arabidopsis*.

Thus in one embodiment of this aspect of the invention, there is disclosed a nucleic acid encoding the polypeptide of Fig 6.

A genomic sequence corresponding to the *Arabidopsis* FRI locus is shown in Fig 4. A putative cDNA sequence transcribed from this genomic sequence is shown at Fig 5. Preferably the nucleic acid of the invention comprises the coding sequence within Fig 5 (bases 362-2188 inclusive). More preferably the nucleic acid comprises the sequence of Fig 5 or Fig 4.

In a further aspect of the present invention there are disclosed nucleic acids which are variants of the sequences provided.

A variant nucleic acid molecule shares homology with, or is identical to, all or part of the coding sequence discussed above. Generally variants may encode, or be used to isolate or amplify nucleic acids which encode, polypeptides which are capable of altering the flowering characteristics of plants as described above. Variants of the present invention can be artificial nucleic acids, which can be prepared by the skilled person in the light of the present disclosure. Alternatively they may be novel, naturally occurring, nucleic

acids, isolatable using the sequences of the present invention.

Sequence variants which occur naturally may include FRI alleles (which will include polymorphisms or mutations at one or more bases) or pseudoalleles (which may occur at closely linked loci to the FRI gene). Also included within the scope of the present invention are isogenes, or other homologous genes belonging to the same family as the FRI gene. Although these may occur at different genomic loci to the gene, they are likely to share conserved regions with it.

Certain allelic and homologous variants are discussed in the Examples below.

Artificial variants (derivatives) may be prepared by those skilled in the art, for instance by site directed or random mutagenesis, or by direct synthesis. Preferably the variant nucleic acid is generated either directly or indirectly (e.g. via one or more amplification or replication steps) from an original nucleic acid having all or part of the sequence shown in Fig 5.

Thus a variant may be a distinctive part or fragment (however produced) corresponding to a portion of the sequence provided. The fragments may encode particular functional parts of the polypeptide. Alternatively, the fragments may have utility in probing for, or amplifying, the sequence provided or closely related ones. Suitable lengths of fragment, and conditions, for such processes are discussed in more detail below.

Also included are nucleic acids corresponding to those above, but which have been extended at the 3' or 5' terminus.

The term 'variant' nucleic acid as used herein encompasses all of these possibilities. When used in the context of

polypeptides or proteins it indicates the encoded expression product of the variant nucleic acid.

5 Some of the aspects of the present invention relating to variants will now be discussed in more detail.

10 Similarity or homology (the terms are used interchangeably) or identity may be as defined and determined by the TBLASTN program, of Altschul et al. (1990) *J. Mol. Biol.* 215: 403-10, or BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). Preferably sequence comparisons are made using FASTA and FASTP (see Pearson & Lipman, 1988. *Methods in Enzymology* 183: 63-98).
15 Parameters are preferably set, using the default matrix, as follows:

20 Gapopen (penalty for the first residue in a gap): -12 for proteins / -16 for DNA
Gapext (penalty for additional residues in a gap): -2 for proteins / -4 for DNA
KTUP word length: 2 for proteins / 6 for DNA.

25 Homology may be at the nucleotide sequence and/or encoded amino acid sequence level. Preferably, the nucleic acid and/or amino acid sequence shares at least about 60%, or 70%, or 80% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% homology or identity.

30 Homology may be over the full-length of the relevant sequence shown herein, or may be over a part of it, preferably over a contiguous sequence of about or greater than about 20, 25, 30, 33, 40, 50, 67, 133, 167, 200, 233, 267, 300, 333, 400 or more amino acids or codons, compared with Fig 6 or 5
35 respectively.

Thus a variant polypeptide in accordance with the present

invention may include within the sequence shown in Fig 6, a single amino acid or 2, 3, 4, 5, 6, 7, 8, or 9 changes, about 10, 15, 20, 30, 40 or 50 changes, or greater than about 50, 60, 70, 80 or 90 changes. In addition to one or more changes within the amino acid sequence shown, a variant polypeptide may include additional amino acids at the C-terminus and/or N-terminus. Naturally, changes to the nucleic acid which make no difference to the encoded polypeptide (i.e. 'degeneratively equivalent') are included.

The activity of functional variant polypeptides may be assessed by transformation into a host capable of expressing the nucleic acid of the invention. Methodology for such transformation is described in more detail below.

In a further aspect of the invention there is disclosed a method of producing a derivative nucleic acid comprising the step of modifying any of the sequences disclosed above, particularly the coding sequence of Fig 5.

Changes may be desirable for a number of reasons. For instance they may introduce or remove restriction endonuclease sites or alter codon usage.

Alternatively changes to a sequence may produce a derivative by way of one or more of addition, insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the addition, insertion, deletion or substitution of one or more amino acids in the encoded polypeptide.

Such changes may modify sites which are required for post translation modification such as cleavage sites in the encoded polypeptide; motifs in the encoded polypeptide for glycosylation, lipoylation etc. Leader or other targeting sequences (e.g. membrane or golgi locating sequences) may be added to the expressed protein to determine its location following expression.

Other desirable mutation may be random or site directed mutagenesis in order to alter the activity (e.g. specificity) or stability of the encoded polypeptide. Changes may be by way of conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. As is well known to those skilled in the art, altering the primary structure of a polypeptide by a conservative substitution may not significantly alter the activity of that peptide because the side-chain of the amino acid which is inserted into the sequence may be able to form similar bonds and contacts as the side chain of the amino acid which has been substituted out. This is so even when the substitution is in a region which is critical in determining the peptides conformation. Also included are variants having non-conservative substitutions. As is well known to those skilled in the art, substitutions to regions of a peptide which are not critical in determining its conformation may not greatly affect its activity because they do not greatly alter the peptide's three dimensional structure. In regions which are critical in determining the peptides conformation or activity such changes may confer advantageous properties on the polypeptide. Indeed, changes such as those described above may confer slightly advantageous properties on the peptide e.g. altered stability or specificity.

In a further aspect of the present invention there is provided a method of identifying and/or cloning a nucleic acid variant from a plant which method employs a FRI sequence described above.

In one embodiment, nucleotide sequence information provided herein may be used in a data-base (e.g. of ESTs, or STSs) search to find homologous sequences, such as those which may become available in due course, and expression products of

which can be tested for activity as described below.

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In another embodiment the nucleotide sequence information provided herein may be used to design probes and primers for probing or amplification of FRI or variants thereof. An oligonucleotide for use in probing or PCR may be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length. For optimum specificity and cost effectiveness, primers of 16-24 nucleotides in length may be preferred. Those skilled in the art are well versed in the design of primers for use processes such as PCR. If required, probing can be done with entire restriction fragments of the gene disclosed herein which may be 100's or even 1000's of nucleotides in length. Naturally sequences may be based on Fig 4 or Fig 5, or the complement thereof. Small variations may be introduced into the sequence to produce 'consensus' or 'degenerate' primers if required.

Such probes and primers form one aspect of the present invention.

Probing may employ the standard Southern blotting technique. For instance DNA may be extracted from cells and digested with different restriction enzymes. Restriction fragments may then be separated by electrophoresis on an agarose gel, before denaturation and transfer to a nitrocellulose filter. Labelled probe may be hybridised to the DNA fragments on the filter and binding determined. DNA for probing may be prepared from RNA preparations from cells. Probing may optionally be done by means of so-called 'nucleic acid chips' (see Marshall & Hodgson (1998) Nature Biotechnology 16: 27-31, for a review).

In one embodiment, a variant in accordance with the present invention is obtainable by means of a method which includes:

(a) providing a preparation of nucleic acid, e.g. from plant cells. Test nucleic acid may be provided from a cell as genomic DNA, cDNA or RNA, or a mixture of any of these, preferably as a library in a suitable vector. If genomic DNA is used the probe may be used to identify untranscribed regions of the gene (e.g. promoters etc.) as described hereinafter,

(b) providing a probe or primer as discussed above,

(c) contacting nucleic acid in said preparation with said nucleic acid molecule under conditions for hybridisation of said nucleic acid molecule to any said gene or homologue in said preparation, and,

(d) identifying said gene or homologue if present by its hybridisation with said nucleic acid molecule. Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include amplification using PCR (see below), RN'ase cleavage and allele specific oligonucleotide probing. The identification of successful hybridisation is followed by isolation of the nucleic acid which has hybridised, which may involve one or more steps of PCR or amplification of a vector in a suitable host.

Preliminary experiments may be performed by hybridising under low stringency conditions. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further.

For example, hybridizations may be performed, according to the method of Sambrook et al. (below) using a hybridization

solution comprising: 5X SSC (wherein 'SSC' = 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50%

5 formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4) 10 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid

15 molecules of a specified sequence homology is (Sambrook et al., 1989): $T_m = 81.5^\circ\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41 (\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\text{\#bp in duplex}$

As an illustration of the above formula, using $[\text{Na}^+] =$
20 $[0.368]$ and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature
25 of 42°C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

It is well known in the art to increase stringency of
30 hybridisation gradually until only a few positive clones remain. Other suitable conditions include, e.g. for detection of sequences that are about 80-90% identical, hybridization overnight at 42°C in 0.25M Na_2HPO_4 , pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X
35 SSC, 0.1% SDS. For detection of sequences that are greater than about 90% identical, suitable conditions include hybridization overnight at 65°C in 0.25M Na_2HPO_4 , pH 7.2, 6.5%

SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

An alternative, which may be particularly appropriate with
5 plant nucleic acid preparations, is a solution of 5x SSPE
(final 0.9 M NaCl, 0.05M sodium phosphate, 0.005M EDTA pH
7.7), 5X Denhardt's solution, 0.5% SDS, at 65°C overnight,
(for high stringency, highly similar sequences) or 50°C (for
10 low stringency, less similar sequences). Washes in 0.2x
SSC/0.1% SDS at 65°C for high stringency, alternatively at
50-60°C in 1x SSC/0.1%SDS for low stringency.

In a further embodiment, hybridisation of nucleic acid
molecule to a variant may be determined or identified
15 indirectly, e.g. using a nucleic acid amplification reaction,
particularly the polymerase chain reaction (PCR). PCR
requires the use of two primers to specifically amplify
target nucleic acid, so preferably two nucleic acid molecules
with sequences characteristic of FRI are employed. Using RACE
20 PCR, only one such primer may be needed (see "PCR protocols;
A Guide to Methods and Applications", Eds. Innis et al,
Academic Press, New York, (1990)).

Thus a method involving use of PCR in obtaining nucleic acid
25 according to the present invention may include:

(a) providing a preparation of plant nucleic acid, e.g. from
a plant cell,

30 (b) providing a pair of nucleic acid molecule primers useful
in (i.e. suitable for) PCR, at least one of said primers
being a primer according to the present invention as
discussed above,

35 (c) contacting nucleic acid in said preparation with said
primers under conditions for performance of PCR,

(d) performing PCR and determining the presence or absence of an amplified PCR product. The presence of an amplified PCR product may indicate identification of a variant.

5 In all cases above, if need be, clones or fragments identified in the search can be extended. For instance if it is suspected that they are incomplete, the original DNA source (e.g. a clone library, mRNA preparation etc.) can be revisited to isolate missing portions e.g. using sequences,
10 probes or primers based on that portion which has already been obtained to identify other clones containing overlapping sequence. As used hereinafter, unless the context demands otherwise, the term "FRI" is intended to cover any of the nucleic acids of the invention described above, including
15 functional variants.

The methods described above may also be used to determine the presence of one of the nucleotide sequences of the present invention within the genetic context of an individual plant,
20 optionally a transgenic plant such as may be produced as described in more detail below. This may be useful in plant breeding programmes e.g. to directly select plants containing alleles which are responsible for desirable traits in that plant species, either in parent plants or in progeny (e.g.
25 hybrids, F1, F2 etc.). Thus use of the newly defined markers disclosed in the Examples below, or markers which can be designed by those skilled in the art on the basis of the nucleotide sequence information disclosed herein, for selection of a gene capable of modifying flowering time in a
30 plant, forms one part of the present invention.

The use of diagnostic tests for alleles allows the researcher or plant breeder to establish, with full confidence and independent from time consuming tests based on actual
35 observation of desired traits (in this case, flowering properties), whether or not a desired allele is present in the plant of interest (or a cell thereof), whether the plant

is a representative of a collection of other genetically identical plants (e.g. an inbred variety or cultivar) or one individual in a sample of related (e.g. breeders' selection) or unrelated plants. In a breeding scheme based on selection and selfing of desirable individuals, nucleic acid or polypeptide diagnostics for the desirable allele or alleles in high throughput, low cost assays as provided by this invention, reliable selection for the flowering time phenotype can be made at early generations and on more material than would otherwise be possible. This gain in reliability of selection plus the time saving by being able to test material earlier and without costly phenotype screening is of considerable value in plant breeding.

Nucleic acid-based determination of the presence or absence of one or more desirable alleles may be combined with determination of the genotype of the flanking linked genomic DNA and other unlinked genomic DNA using markers such as RFLPs, microsatellites or SSRs, AFLPs, RAPDs etc which are either very closely linked to FRI locus, as shown in the Examples hereinafter, or are adapted to identify individual FRI alleles for direct allele selection. This enables the researcher or plant breeder to select for not only the presence of the desirable allele but also for individual plant or families of plants which have the most desirable combinations of linked and unlinked genetic background. Such recombinations of desirable material may occur only rarely within a given segregating breeding population or backcross progeny. Direct assay of the locus as afforded by the present invention allows the researcher to make a step-wise approach to fixing (making homozygous) the desired combination of flanking markers and alleles, by first identifying individuals fixed for one flanking marker and then identifying progeny fixed on the other side of the locus all the time knowing with confidence that the desirable allele is still present.

In a further aspect of the present invention, nucleic acid encoding FRI is in the form of a recombinant and preferably replicable vector.

5 "Vector" is defined to include, inter alia, any plasmid, cosmid, phage or *Agrobacterium* binary vector in double or single stranded linear or circular form which may or may not be self transmissible or mobilizable, and which can transform a prokaryotic or eukaryotic host either by integration into
10 the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication).

Generally speaking, those skilled in the art are well able to
15 construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other
20 sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press or *Current Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992.

25 Specifically included are shuttle vectors by which is meant a DNA vehicle capable, naturally or by design, of replication in two different host organisms, which may be selected from actinomycetes and related species, bacteria and eucaryotic
30 (e.g. higher plant, mammalian, yeast or fungal cells).

A vector including nucleic acid according to the present invention need not include a promoter or other regulatory sequence, particularly if the vector is to be used to
35 introduce the nucleic acid into cells for recombination into the genome.

Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate promoter or other regulatory elements for transcription in a host cell such as a microbial, e.g. bacterial, or plant cell. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of FRI genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

In a preferred embodiment, the promoter is an inducible promoter.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct

stimulus.

Thus this aspect of the invention provides a gene construct, preferably a replicable vector, comprising a promoter
5 (optionally inducible) operably linked to a nucleotide sequence provided by the present invention, such as the FRI gene or a variant thereof.

Particular of interest in the present context are nucleic
10 acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS
15 Scientific Publishers, pp 121-148). Suitable vectors may include plant viral-derived vectors (see e.g. EP-A-194809).

Suitable promoters which operate in plants include the Cauliflower Mosaic Virus 35S (CaMV 35S). Other examples are
20 disclosed at pg 120 of Lindsey & Jones (1989) "Plant Biotechnology in Agriculture" Pub. OU Press, Milton Keynes, UK. The promoter may be selected to include one or more sequence motifs or elements conferring developmental and/or tissue-specific regulatory control of expression. Inducible
25 plant promoters include the ethanol induced promoter of Caddick et al (1998) Nature Biotechnology 16: 177-180.

If desired, selectable genetic markers may be included in the construct, such as those that confer selectable phenotypes
30 such as resistance to antibiotics or herbicides (e.g. kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate).

35 The present invention also provides methods comprising introduction of such a construct into a plant cell or a microbial cell and/or induction of expression of a construct

within a plant cell, by application of a suitable stimulus e.g. an effective exogenous inducer.

The invention further encompasses a host cell transformed with nucleic acid or a vector according to the present invention (e.g comprising the FRI sequence) especially a plant or a microbial cell. In the transgenic plant cell (i.e. transgenic for the nucleic acid in question) the transgene may be on an extra-genomic vector or incorporated, preferably stably, into the genome. There may be more than one heterologous nucleotide sequence per haploid genome.

The term "heterologous" is used broadly in this aspect to indicate that the gene/sequence of nucleotides in question (e.g. encoding FRI) have been introduced into said cells of the plant or an ancestor thereof, using genetic engineering, i.e. by human intervention. A heterologous gene may replace an endogenous equivalent gene, i.e. one which normally performs the same or a similar function, or the inserted sequence may be additional to the endogenous gene or other sequence. Nucleic acid heterologous to a plant cell may be non-naturally occurring in cells of that type, variety or species. Thus the heterologous nucleic acid may comprise a coding sequence of or derived from a particular type of plant cell or species or variety of plant, placed within the context of a plant cell of a different type or species or variety of plant. A further possibility is for a nucleic acid sequence to be placed within a cell in which it or a homologue is found naturally, but wherein the nucleic acid sequence is linked and/or adjacent to nucleic acid which does not occur naturally within the cell, or cells of that type or species or variety of plant, such as operably linked to one or more regulatory sequences, such as a promoter sequence, for control of expression.

Thus a further aspect of the present invention provides a method of transforming a plant cell involving introduction of

a construct as described above into a plant cell and causing or allowing recombination between the vector and the plant cell genome to introduce a nucleic acid according to the present invention into the genome.

The host cell (e.g. plant cell) is preferably transformed by the construct, which is to say that the construct becomes established within the cell, altering one or more of the cell's characteristics.

Nucleic acid can be introduced into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614 Gelvin Debeyser) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman et al. *Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species.

Recently, there has also been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (see e.g. Hiei et al. (1994) *The Plant Journal* 6, 271-282)). Microprojectile bombardment, electroporation and direct DNA uptake are preferred where *Agrobacterium* alone is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the

transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

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The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention.

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Generally speaking, following transformation, a plant may be regenerated, e.g. from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989.

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The generation of fertile transgenic plants has been achieved in the cereals rice, maize, wheat, oat, and barley (reviewed in Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5, 158-162.; Vasil, et al. (1992) *Bio/Technology* 10, 667-674; Vain et al., 1995, *Biotechnology Advances* 13 (4): 653-671; Vasil, 1996, *Nature Biotechnology* 14 page 702).

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Plants which include a plant cell according to the invention are also provided.

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In addition to the regenerated plant, the present invention embraces all of the following: a clone of such a plant, seed, selfed or hybrid progeny and descendants (e.g. F1 and F2

descendants).

A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the plant or an ancestor thereof.

The invention also provides a plant propagule from such plants, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. It also provides any part of these plants e.g. edible leaves which includes the plant cells or heterologous DNA described above.

Preferred plants which may be transformed with FRI (thereby delaying flowering time) include sugar beet, Brassicas (especially cauliflower, broccoli, cabbage, spinach, curly kale, *B. Napus*) and potato, lettuce and culinary herbs.

It may be preferable to use FRI in conjunction with other genes or mutations which affect the flowering time of plants. Examples include use of the FLC gene, which may enhance the effects the FRI gene.

In particular it may be preferred to employ FRI in plants which exhibit a reduced vernalization response. One means by which this may be achieved is by means of the VRN2 gene. VRN2 is believed to mediate the vernalisation response in plants. Down-regulation of VRN2 expression, for instance by means of an antisense VRN2 cDNA, may therefore be used to inhibit the effect of vernalisation in promoting flowering. VRN2 cDNA sequences from *Arabidopsis thaliana* Landsberg erecta and Columbia are shown after the References. Anti-sense methodology is discussed below.

The invention further provides a method of influencing or affecting a flowering time in a plant, the method including the step of causing or allowing expression of a heterologous FRI nucleic acid sequence as discussed above within the cells of the plant.

The invention further provides a method comprising the step of causing or allowing expression of a nucleic acid encoding FRI or a variant thereof, within cells of a plant (thereby producing the encoded polypeptide) such as to alter the flowering time.

The step may be preceded by the earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof.

The foregoing discussion has been generally concerned with uses of the nucleic acids of the present invention for production of functional polypeptides, thereby increasing the flowering time of the plant.

However the information disclosed herein may also be used to reduce the activity of FRI in cells in which it is desired to do so, thereby having the opposite effect.

Accelerating flowering time may be useful in certain species e.g. soft fruits such as strawberries, raspberries, or crops such as maize.

Down-regulation of FRI expression may, for instance, be achieved using anti-sense technology.

In using anti-sense genes or partial gene sequences to down-regulate gene expression, a nucleotide sequence is placed under the control of a promoter in a "reverse orientation" such that transcription yields RNA which is complementary to normal mRNA transcribed from the "sense"

strand of the target gene. See, for example, Rothstein et al, 1987; Smith et al, (1988) *Nature* 334, 724-726; Zhang et al, (1992) *The Plant Cell* 4, 1575-1588, English et al., (1996) *The Plant Cell* 8, 179-188. Antisense technology is also
5 reviewed in Bourque, (1995), *Plant Science* 105, 125-149, and Flavell, (1994) *PNAS USA* 91, 3490-3496.

Thus a nucleotide sequence which is complementary to any of those coding sequences disclosed above forms one part of the
10 present invention.

An alternative to anti-sense is to use a copy of all or part of the target gene inserted in sense, that is the same, orientation as the target gene, to achieve reduction in
15 expression of the target gene by co-suppression. See, for example, van der Krol et al., (1990) *The Plant Cell* 2, 291-299; Napoli et al., (1990) *The Plant Cell* 2, 279-289; Zhang et al., (1992) *The Plant Cell* 4, 1575-1588, and US-A-5,231,020. Further refinements of the gene silencing or co-
20 suppression technology may be found in WO95/34668 (Biosource); Angell & Baulcombe (1997) *The EMBO Journal* 16,12:3675-3684; and Voinnet & Baulcombe (1997) *Nature* 389: pg 553.

25 Further options for down regulation of gene expression include the use of ribozymes, e.g. hammerhead ribozymes, which can catalyse the site-specific cleavage of RNA, such as mRNA (see e.g. Jaeger (1997) "The new world of ribozymes" *Curr Opin Struct Biol* 7:324-335, or Gibson & Shillitoe
30 (1997) "Ribozymes: their functions and strategies form their use" *Mol Biotechnol* 7: 242-251.)

The complete sequence corresponding to the coding sequence (in reverse orientation for anti-sense) need not be used.
35 For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of

the coding sequence to optimise the level of anti-sense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a conserved sequence of a gene, e.g. a sequence that is characteristic of one or more genes, such as a regulatory sequence.

The sequence employed may be about 500 nucleotides or less, possibly about 400 nucleotides, about 300 nucleotides, about 200 nucleotides, or about 100 nucleotides. It may be possible to use oligonucleotides of much shorter lengths, 14-23 nucleotides, although longer fragments, and generally even longer than about 500 nucleotides are preferable where possible, such as longer than about 600 nucleotides, than about 700 nucleotides, than about 800 nucleotides, than about 1000 nucleotides or more.

It may be preferable that there is complete sequence identity in the sequence used for down-regulation of expression of a target sequence, and the target sequence, although total complementarity or similarity of sequence is not essential. One or more nucleotides may differ in the sequence used from the target gene. Thus, a sequence employed in a down-regulation of gene expression in accordance with the present invention may be a wild-type sequence (e.g. gene) selected from those available, or a variant of such a sequence.

The sequence need not include an open reading frame or specify an RNA that would be translatable. It may be preferred for there to be sufficient homology for the respective anti-sense and sense RNA molecules to hybridise. There may be down regulation of gene expression even where there is about 5%, 10%, 15% or 20% or more mismatch between the sequence used and the target gene. Effectively, the homology should be sufficient for the down-regulation of gene expression to take place.

Thus the present invention further provides the use of the nucleotide sequence of Fig 5, or its complement, or a variant of either for down-regulation of gene expression, particularly down-regulation of expression of the FRI gene or a homologue thereof, preferably in order to influence (accelerate) the flowering time of a plant.

Anti-sense or sense regulation may itself be regulated by employing an inducible promoter in an appropriate construct.

Although the above description has generally been concerned with the transcribed parts of the FRI gene (in sense or antisense) and variants and products thereof, also embraced within the present invention are untranscribed parts of the gene. Thus a further aspect of the invention is a nucleic acid molecule encoding the promoter or other transcriptional control sequence of the FRI gene.

The promoter region or other control sequences may be readily identified on the basis of the genomic sequence shown in Fig 4 using a probe or primer as described above in relation to the isolation of variants. Generally they will be found 5' to the open reading frame of the gene and are obtainable by probing a genomic DNA library with a nucleic acid of the invention, selecting a clone which hybridizes under conditions of medium to high stringency, and sequencing the clone 5' to the open reading frame of the gene. Where only a small amount of sequence is present in the 5' region, this sequence may be used to reprobe the library to genome walk further upstream. Analysis of the upstream region will reveal control regions for gene expression including control regions common to many genes (i.e TATA and CAAT boxes) and other control regions, usually located from 1 to 10,000, such as 1 to 1000 or 50 to 500 nucleotides upstream of the start of transcription. Sequences identified as described above can be assessed for promoter activity.

"Promoter activity" is used to refer to ability to initiate transcription. The level of promoter activity is quantifiable for instance by assessment of the amount of mRNA produced by transcription from the promoter or by assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which are able to hybridise with the mRNA and which are labelled or may be used in a specific amplification reaction such as the polymerase chain reaction.

Use of a reporter gene facilitates determination of promoter activity by reference to protein production. The reporter gene preferably encodes an enzyme which catalyses a reaction which produces a detectable signal, preferably a visually detectable signal, such as a coloured product. Many examples are known, including β -galactosidase and luciferase.

Those skilled in the art are well aware of a multitude of possible reporter genes and assay techniques which may be used to determine promoter activity. Any suitable reporter/assay may be used and it should be appreciated that no particular choice is essential to or a limitation of the present invention.

Thus in one aspect of the invention there is provided a nucleic acid construct, preferably an expression vector, including the FRI promoter region or fragment operably linked to a heterologous gene, e.g. a coding sequence, which is preferably not the coding sequence with which the promoter is operably linked in nature.

The present invention also encompasses the expression product of any of the coding (sense) nucleic acid sequences disclosed above, and methods of making the expression product by expression from encoding nucleic acid therefore under

suitable conditions, which may be in suitable host cells.

Following expression, the product may be isolated from the expression system (e.g. microbial) and may be used as
5 desired.

The present invention particularly provides for the production and use of fragments of the full-length polypeptides disclosed herein, especially active portions
10 thereof. An "active portion" of a polypeptide means a peptide which is less than said full length polypeptide, but which retains an essential biological activity. In particular, the active portion retains the ability to alter flowering time in a plant such as *Arabidopsis thaliana*.

15 A "fragment" of a polypeptide means a stretch of amino acid residues of at least about five to seven contiguous amino acids, often at least about seven to nine contiguous amino acids, typically at least about nine to 13 contiguous amino
20 acids and, most preferably, at least about 20 to 30 or more contiguous amino acids. Fragments of the polypeptides may include one or more epitopes useful for raising antibodies to a portion of any of the amino acid sequences disclosed herein. Preferred epitopes are those to which antibodies are
25 able to bind specifically, which may be taken to be binding a polypeptide or fragment thereof of the invention with an affinity which is at least about 1000x that of other polypeptides.

30 For instance, purified FRI protein, or a variant thereof, e.g. produced recombinantly by expression from encoding nucleic acid therefor, may be used to raise antibodies employing techniques which are standard in the art.

Antibodies and polypeptides comprising antigen-binding
35 fragments of antibodies may be used in identifying homologues from other species as discussed further below.

Methods of producing antibodies include immunising a mammal (e.g. human, mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and might be screened, preferably using binding of antibody to antigen of interest.

For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82).

Antibodies may be polyclonal or monoclonal.

Antibodies may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any specific binding substance having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of Chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023. It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are well known to those skilled in the art.

As an alternative or supplement to immunising a mammal, antibodies with appropriate binding specificity may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047.

Antibodies raised to a polypeptide or peptide can be used in

the identification and/or isolation of variant polypeptides, and then their encoding genes. Thus, the present invention provides a method of identifying or isolating a FRI polypeptide or variant thereof (as discussed above),

5 comprising screening candidate polypeptides with a polypeptide comprising the antigen-binding domain of an antibody (for example whole antibody or a fragment thereof) which is able to bind said FRI polypeptide or variant thereof, or preferably has binding specificity for such a
10 polypeptide.

Specific binding members such as antibodies and polypeptides comprising antigen binding domains of antibodies that bind and are preferably specific for a FRI polypeptide or mutant
15 or derivative thereof represent further aspects of the present invention, as do their use and methods which employ them.

The invention will now be further described with reference to
20 the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

FIGURES

25 Fig 1 shows a physical map of the genomic region covering the *FRI* from the Arabidopsis Columbia ecotype based on a YAC/BAC contig.

30 Fig 2 shows a cosmid contig covering *FRI*, with the complementing cosmid (84M13) shown shaded.

Fig 3 shows various subclones of 84M13 which were used to narrow down the *FRI* region within the cosmid.

35 Fig 4 shows the (late flowering) H51 genomic sequence of the first 17 kb of 84M13 encompassing the *FRI* gene.

Fig 5 shows the likely cDNA sequence of the H51 FRI gene.

Fig 6 shows the FRI amino acid sequence predicted from the cDNA sequence.

Fig 7 shows Table 3.

EXAMPLES

Example 1- Mapping and isolation of the FRIGIDA gene

Genetic fine mapping of the FRI locus.

Co-segregation analysis of *FRI* with markers mi51, mi122, mi204 and g3843 had mapped the *FRI* locus near the top of chromosome 4 (Clarke and Dean, 1994). These and additional markers, derived from the physical map of chromosome 4 (Schmidt et al., 1995) were then used to fine-map the locus. 590 F₂ plants from a cross between the late flowering parent H51 (Napp-Zinn, 1957; Napp-Zinn, 1962) and the early flowering parent Li5 were scored for their flowering time. 344 of the plants flowered at the same time as H51 (late) and 146 plants flowered at the same time as Li-5 (early). This 3:1 ratio of late to early flowering time confirmed that *FRI* segregates as a single locus with late flowering being the dominant phenotype. The early flowering F₂ plants were allowed to self. The resulting F₃ plants were scored for their flowering time to confirm that they were homozygous for the Li-5 allele at *FRI*. The segregation of the four markers described above, closely linked to *FRI*, was then scored in the 146 F₂ early flowering plants. DNA from the F₃ families was digested with the restriction endonucleases that gave polymorphisms between H51 and Li-5 and Southern blots were prepared. These were then probed with the genetic markers. Since all the F₃ plants had the Li-5 allele at the *FRI* locus and the DNA markers had been found to be either closely or tightly linked to *FRI* it was anticipated that most of the F₃

plants would show the Li-5 hybridisation patterns on the resulting autoradiographs. However, in a few of the plants there may have been a recombination event very close to *FRI*. These plants would then show a heterozygous pattern with one or more of the DNA markers. Two recombinant plants were found in the population of 146 early flowering plants. Plant number 156 (p156) showed a heterozygous pattern with marker mi51, but a Li5 pattern with marker g8802, mi204, mi122 and g3843. Plant number 5 (p5) showed a heterozygous pattern with marker g3843 but a Li5 pattern with marker mi122, mi204 and mi51. These data placed *FRI* between mi51 and g3843 on chromosome 4, a genomic region of 350kb.

A further 180 early flowering F2 plants from an H51 X Li-5 cross were screened with both mi51 and g3843 (both markers showed a polymorphism between H51 and Li-5 with the restriction endonuclease *Dra* I). In addition, 78 plants were scored with mi51 only and 29 plants were scored with g3843 only. A total of five recombinant plants resulted from this screen; three plants showed a heterozygous pattern with mi51 and two plants showed a heterozygous pattern with g3843. These recombinant plants were checked to see if they gave a heterozygous pattern with other markers in the region and between which interval the recombination event had occurred. One that was heterozygous for g3843 was also heterozygous for CC27P11 so defining the location of *FRI* to an interval of 320kb.

Physical mapping of FRI.

The 320 kb region on the top of chromosome 4 between RFLP markers mi51 and CC27P11 was covered by a physical map comprising of yeast artificial chromosome (YAC) clones (Clarke and Dean, 1994). Bacterial artificial chromosome clones (BACs) were integrated into this map through the efforts of scientists at Cold Spring Harbor Laboratory USA (<http://www.cshl.org/protarab/chrom4map.htm>) and Washington University USA

(<http://genome.wustl.edu/gsc/arab/arabidopsis.html>). The physical map of the genomic region covering *FRI* from the Arabidopsis Columbia ecotype is shown in Fig.1

5 *Complementation and further mapping.*

10 The genetics of the flowering time suggested that late
flowering ecotypes contained functional *FRI* alleles whereas
early flowering ecotypes contained only partially functional
or non-functional alleles. The libraries available for
15 complementation experiments had all been generated from early
flowering ecotypes (Landsberg *erecta*, Columbia and WS). In
order to do complementation experiments for *FRI*, a cosmid
library had to be made from an ecotype carrying a dominant
20 *FRI* allele. H51 DNA partially digested with *Sau3A* was cloned
into a cosmid vector (Clare Lister and C Dean unpublished)
that also carried *Agrobacterium* LB and RB T-DNA sequences and
a 35S-NPTII-ocs3' plant selectable marker (CLD04541). 60000
clones were picked into 384 well microtitre plates. In theory
25 there should have been more than a ten-fold redundancy in
this library. The library was hybridized with yeast
artificial chromosome clones (YACs) CIC11A2, EW19A10, EW
12E9, EW 13F8, EW 10A8 and bacterial artificial chromosome
clones (BACs) IGF 5I10, IGF 6N23, TAMU18A10 and IGF2N01
30 covering the *FRI* region (Fig. 1). 93 positively hybridizing
cosmid clones were identified. 59 of these clones were
confirmed by fingerprinting and Southern blots to be unique
and positioned between markers mi51 and CC27P11. The 59
cosmids were then placed into "bins" by hybridization with
35 overlapping BAC clones TAMU8M24, TAMU29E23 and TAMU 10K22.
In addition, the sequence of this genomic region started to
become available as our analysis was proceeding. We then
used the Columbia (Col) genomic sequence as a scaffold to
assemble the H51 cosmid clones into contigs (eg Fig 2). All
59 cosmid clones were anchored onto the BAC sequence by end
sequencing the cosmid insert and comparing it to the Columbia
sequence. This analysis determined the orientation of the

BACs relative to each other and relative to the chromosome. The colinearity of the H51 line and Col was investigated by comparison of restriction fragments from the cosmids to the map generated from the BAC sequence. Three cosmid contigs
5 corresponding to BACs F5I10, F6N23 and T18A10 were assembled in this manner.

The cosmid library has been constructed in the SURE™ tet^S *E. coli* strain (Stratagene) to avoid deletions in unstable clones. One drawback of the SURE *E. coli* strain is
10 the poor conjugation with *Agrobacterium*. All 59 clones were transformed into *E. coli* DH5αF' and then conjugated using triparental mating into *Agrobacterium*. After each step all clones were verified to be identical to the original clone by restriction fragment analysis.

15 The cosmids were introduced into early flowering *Arabidopsis* Li5 plants using the vacuum-infiltration *Agrobacterium*-transformation procedure (Bechtold et al., 1993). Over 300 transformants were obtained with the aim of 5 independent transformants for each cosmid clone.

20 As the complementation experiments proceeded, further mapping was undertaken. The markers mi51,122 and 204 were anchored onto the BAC contig by sequencing their inserts. Two new RFLP markers using H51 cosmids 40D10 and 32F5 were also developed. The alignment of H51 cosmids to the Col
25 sequence enabled the identification of polymorphic regions and construction of 8 PCR markers in the *FRI* region (vertical bars, Fig.1 and Table 1). The SSLP marker, UJ3-4 was sensitive enough to identify one heterozygous recombinant plant in a tissue pool of 10 plants. This allowed the
30 isolation of five new recombinant plants by screening batches of 100 plants with just 20 minipreps and 20 PCRs. The CAPS marker UJ12-13 at the other end is less sensitive and was combined with a Southern blot to ensure the detection of recombinants in the same pools. These two markers made it
35 possible to screen large populations of plants to isolate a lot of new recombinants with very little effort.

PCR markers were also used to fine map the recombination

break points in the 7 previously identified recombinants. This allowed the *FRI* region to be shortened ~ 75 kb on centromere side, eliminating the BAC 2N01 and a gap in the contig. 50 kb on the telomere side of the *FRI* locus has also been excluded, thereby reducing the number of cosmid clones for the complementation experiment to a total of 33.

Initially only the clone 84M13 was found to complement in the T1 generation (Fig. 2). Transformations with overlapping clones and subclones were pursued in order to narrow down the *FRI* region below the 21 kb of 84M13 (Fig. 3). Of the overlapping clones only 41J6 could complement whereas 47L16, 163M11 and 23N15 could not. (For some reason 41J6 seems to have much lower transformation efficiency than 84M13.) Some subclones of 84M13; pJU226, pJU222 and pJU223, do also complement whereas pJU234, pJU225 and pJU235 do not. The complementing cosmid pJU226 contains two copies of an *EcoRI*- fragment in a tandem repeat, but it is likely that at least most of the *FRI* gene is contained within a 5.5 kb *XbaI*-*EcoRI* fragment in the middle of 84M13.

Example 2 - The sequence of FRIGIDA.

Sub 39

The H51 genomic sequence of the first 17 kb of 84M13 has been determined (*FRI* sequence in Fig.4). RT-PCR experiments have been used to delimit the transcribed region using primers described in Table 2. Products were obtained using the primers at the 5' end CLFRI10 -UJ43 but not when CLFRI9 -UJ43 were used. This predicts the start of transcription to be between CLFRI-9 and CLFRI10 and there is a putative TATA box 20bp downstream of CLFRI9. The primers CLFRI11, CLFRI 1, CLFRI2, CLFRI3 all gave products with UJ43 that were the same size from cDNA made from polyA RNA isolated from H51 plants as from plasmid DNA indicating that there were no introns in this region. This then places the beginning of the open reading frame downstream of a stop codon at position 455 (Fig. 4). The first MET codon is at position 574 (Fig 4) giving a 5' untranslated leader of >360 nucleotides. By using

RT-PCR we were able to verify that this gene is transcribed at least in leaves of both Li-5 and H51. Furthermore we were able to amplify the cDNA with primers UJH-UJ37 and to verify the positions of the first two introns by digestions of RT-PCR products (the second splice site creating a *Dra*I restriction site). The next stop-codon in frame (2880, Fig. 4) is likely to terminate translation. This stop-codon is well before the *Eco*RI site (15516) which defines the end of the complementing *FRI* gene in pJU226. RT-PCR analysis of the 3' end of the transcribed region yielded a product between UJ30 and an oligo dT primer of ~540bp putting the polyadenylation site around the primer CLFRI16. This would give a 3' untranslated region of ~500 nucleotides. The most likely transcribed sequence is shown in Fig. 5, the exact delimitation at both the 5' and 3' ends has still not been determined. The predicted amino acid sequence from this cDNA is shown in Fig. 6.

Loss of function FRI alleles.

An EMS mutagenesis of H51 was undertaken to identify loss of function alleles of *FRI*. All 40 early flowering mutants identified were crossed to Li5 to test for allelism with *FRI*. None however were allelic.

In a separate study, about 70 early flowering mutants were isolated from another late ecotype, Sf-2, by fast neutron radiation. Allelism tests suggested four might be allelic to *FRI*. A preliminary study of hybridizing BAC DNA onto DNA isolated from the early lines suggested that 5 of these FN-mutant lines had deletions in a 15 kb region within the cosmid clones 84M13 and 41J6. We have been able to complement 3 of these mutants: FN13, FN233 and FN235 with 84M13, thereby confirming that the mutations causing the early flowering are indeed in this region. The FN mutants were analyzed by Southern. FN233 and FN235 were confirmed to have large deletions in the region corresponding to 84M13, resulting in lines with intact promoter regions but lacking most of the coding part of the *FRI* gene (Fig.3). FN13 could

be interpreted as carrying a recombination or a large insertion at the very beginning of *FRI* (Fig.3). The analysis of the FN mutants and the subclones of 84M13 support the notion that dominant late flowering alleles of *FRI* are expressing an active protein whereas the early lines have non-functional alleles of *FRI*.

Allelic variation at FRIGIDA.

When the sequence of the H51 *FRI* allele is compared to that in Col there are 6 nucleotide substitutions in the *FRI* region. The first two (positions 398 and 432) are in the 5'UTR. Two result in amino acid changes (1010 G in H51, A in Col) and 1017 (G in H51, A in Col) changing Gly to Glu and Met to Ile. A fifth substitution is found in the middle of the first intron and the last is a synonymous substitution at position 1971. The largest difference between the H51 and the Col sequence is a 16 bp deletion in Col (1509-1524). This deletion is in the first exon of *FRI* just before the donor splice and would result in a change of reading frame in Col relative to H51. The frame shift would lead to translational termination at a stop codon in the next exon and synthesis of a protein about half the size of the predicted H51 allele.

In order to characterize the *FRI* alleles of early and late ecotypes two PCR markers were constructed. These markers allowed us to investigate if the Gly polymorphism (generation of a BsmFI site), or the 16 bp deletion occurred in other ecotypes (Table 3). The late ecotypes fell into two classes and either showed the same pattern as H51, or contained the Gly polymorphism. The early flowering ecotypes fell into two classes based on the use of these markers. One class of early ecotypes looked like Col, ie. they had both the changes relative to H51. Another class of plants looked like H51 with the two markers, but consisted of early and intermediate ecotypes. The early *FRI* allele from one member of this class, Ler, was sequenced to find out if there were

other mutations in the gene. A deletion of 375 bp and insertion of a novel 31bp was found at the 5' end of the open reading frame of the Ler *FRI* allele. This deletion would abolish the likely translation initiation codon, thereby probably creating a null allele of *FRI*. A perfect match to the first 19 bp of these 31 bp is found 64 bp 3' of the insertion. 9 bp of the 19 bp repeat is also found in the second half (12 bp) of the 31 bp insertion. It is quite plausible that the insertion was created at the same time as the deletion arose and tells something about how the deletion occurred and/or how the DNA lesion was repaired. The 31 bp insertion contains an ATG codon so translation may initiate but would result in a short peptide in a different frame to *FRI*. There were only two other differences between the Ler sequence and that from H51. One is the same substitution that is found in an intron in the Col sequence. The other change would have resulted in an amino acid change if the promoter region of Ler was intact. However, this is a conserved substitution of Leu to Ile. It is plausible that both these changes took place before the deletion inactivated the gene.

When the ecotypes were re-examined in the promoter region with PCR, this mutation was also found in the ecotypes Dijon and Gr. The finding of the promoter deletion in other ecotypes beside Ler, argues that it was present in the Ler ancestor and is not the result of any recent mutagenesis of the Ler parent. It is possible that Dijon and Gr contain a late *FLC* allele unlike Ler and that this difference is responsible for their intermediate flowering time.

A third group of early flowering ecotypes do not contain either the 16bp deletion present in Col or the larger deletion present in Ler.

It is possible that representatives from this class will contain further polymorphisms. From the results above it appears that there are at least two different *FRI* alleles that confer late flowering. There are at least two different alleles of *FRI* that result in early flowering, that appear to

be *FRI* loss of function alleles and that have arisen independently of each other from a late allele of *FRI*. It is interesting to note that the ancestor of Arabidopsis was probably late flowering, carrying a functional allele of *FRI*. This makes it likely that an orthologue of *FRI* will be found in related species and might also form the genetic basis for variation in flowering time among them in the same manner it does in Arabidopsis.

10 Example 3 - Predicting FRIGIDA function.

Database predictions show *FRI* to be an alpha helical protein with no membrane spanning domains. Database searches of the protein sequence against the Arabidopsis protein set revealed two related predicted proteins from elsewhere in the Arabidopsis genome. One is on BAC TAMU21B4 on chromosome 5 and is annotated as having similarity to IP3 receptor. It shares 26% amino acid identity and 51% similarity (over 297 amino acids). However when the sequence from TAMU21B4 was run against all available databases using BLASTP no protein with homology to IP3 was found so the basis of the annotation is unclear. The second protein mapping to the ESSA I No2 fragment on chromosome 4 is annotated as being a hydroxyproline rich glycoprotein homologue. It shares 21% amino acid identity and 42% similarity over 236 amino acids. *FRI* however does not contain the high proportion of proline residues characteristic of this family of proteins. These proteins are unlikely to represent *FRI* functional homologues.

30 A TBLASTN analysis against the Arabidopsis Genbank dataset gave the same proteins from the Arabidopsis genome sequence as described above. In addition a few EST sequences were detected with very low homology scores. For example EST clone 3D1T7P showed the low score of $p=0.00031$ and shared 29% amino acid identity over only 107 amino acids. No EST corresponding to the *FRI* Col allele is present in the databases. A BLASTP analysis against the non-redundant

SwissProt database showed a range of functionally unrelated proteins all with very low homology scores ($e > 0.68$). A proportion of the proteins were however related in some way to the cytoskeleton: yeast intracellular protein transport protein USO1, NUF1 spindle body spacer protein, troponin T etc. This precipitated us to look more closely at the secondary structure of FRI. Using programmes available on the Internet, provided by Andrei Lupas, FRI is predicted to contain two coiled coil domains (between amino acids 45-100 and 400-450). Coiled coil domains are common in cytoskeletal proteins as they cause the protein to have a rod-like configuration. They are a hall mark of protein: protein interactions. Proteins either homo or heterodimerize through the coiled coil regions (Cohen and Parry, 1986; Lupas et al., 1991).

Example 4 - FRIGIDA homologues.

The database searches had revealed the existence of *FRI* homologues showing relatively weak homology (in the order of 26% amino acid identity) to *FRI*. However, the presence of cross-hybridizing fragments in addition to *FRI* on genomic Southern blots that had been washed at high stringency suggests that there is at least one closely related *FRI* homologue in Arabidopsis not yet represented in the databases. Hybridization of a genomic fragment (which also contains an overlapping superoxide dismutase homologue transcribed on the complementary strand) to the yeast artificial chromosome clones that constitute the Arabidopsis physical map shows that a potential *FRI* homologue maps to YAC clones CIC1F8 and 4H4.

Example 5 - production of late-flowering transgenic potato

Premature bolting in potato diverts the partitioning of assimilates from the tuber towards have an extended vegetative phase are desirable over those which do not.

This significantly reduces tuber yield. Accordingly, plants which have an extended vegetative phase are desirable over those which do not.

- 5 An FRI genomic clone, or FRI sequence operably linked to the CaMV 35S promoter (Odell et al, 1985 Nature 313, 810-812) is introduced into potato plants according to the method of Spychalla and Bevan (1993) Plant Tissue Culture Manual B11 1-9. Plants are observed having an extended vegetative phase.

10 As an alternative, an additional construct comprising VRN2 in antisense operably linked to the CaMV 35S promoter is also introduced into a plant. A decreased vernalisation response, and delayed flowering time results.

TABLES

Table 1

| 20 | <u>MARKER</u> | <u>CLOSE RFLP</u> <u>POSITION</u> | <u>BAC</u> | <u>PRIMER</u> | <u>SEQUENCE</u> |
|----|---------------|--------------------------------------|---------------|---------------|---------------------------------|
| | UJ3-4 | CC27P11 | F2N01 | UJ3 | GTC GGA CCA CAG TTG ATA AGA AT |
| | | | 98105-100090 | UJ4 | TCG CAG ATA AGG AGA CTA ACC A |
| 25 | UJ8-9 | 40D10 | T18A10 | UJ8 | GAG TTC CGC GAC CCT TTA C |
| | | | 47043-472167 | UJ9 | TAG TTT CCG TTG ATA TGT GAT TT |
| 30 | UJ10-11 | mi122 | T18A10 | UJ10 | TAA GAA GCC GAA AAC AAA AGG AT |
| | | | 90704-91517 | UJ11 | AGG GTA AAA ACT GCA GAT GAA AAT |
| | UJ12-13 | mi51 | F5I10 | UJ12 | CGG GGT CAG GTA ATA GCA CAC |
| | | | 105629-106219 | UJ13 | GGT TTT CGG ATT TCG GAT TTT A |
| 35 | UJ14-15 | mi51-mi204 | F5I10 | UJ14 | AAT TCA ACC GCA TCG TAT CAG |
| | | | 54494-55506 | UJ15 | TAT CAG CCG TAT CAA CCA CAT T |

| | | | | |
|---------|-------------|-------------|------|---------------------------------|
| UJ18-19 | mi51-mi204 | F5I10 | UJ18 | CCA CCG TTA GTC TAT GCC TGA GTA |
| | | 49820-50835 | UJ19 | GAT GGG TCG GTG GGT GAA C |
| UJ20-2 | mi204-mi122 | F6N23 | UJ20 | ACC GCA GAA GCA GCA TTA GC |
| | | 52046-53173 | UJ21 | GTC CGC GCA GGT GAT TTG |
| UJ24-25 | mi204-mi122 | F6N23 | UJ24 | CTC CCG ACA GTT TCT TTG ACG |
| | | 45586-47168 | UJ25 | CCT GTT CCT GGC GGT GTA G |

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Table 1 (continued)

| | UJG | CTA | AAC | ATA | TAA | CGA | TTA | CC | 386 > 405 |
|----|------------|-----|--------|-----|-----|-----|-----|-----------|-------------|
| | UJ41 | CGT | TTT | CTC | CTA | ATT | AAA | AG | |
| 5 | UJ41-2 | CGT | TTT | CTC | CTA | CTT | AAA | AG | 420 > 439 |
| | CLFRI-2 | CTT | CAC | AAT | ATA | CAG | TTC | A | 477 > 495 |
| | CLFRI-3 | GTG | GAA | ATT | AGG | GCT | TCT | G | 529 > 547 |
| 10 | RI-CLFRI-3 | CCA | GAATTC | GTG | GAA | ATT | AGG | GCT TCT G | 529 > 547 |
| | UJP | GTG | GAT | AAT | TGG | ACA | TGA | G | 589 > 571 |
| 15 | UJH | CCA | TAG | ACG | AAT | TAG | CTG | C | 746 > 764 |
| | UJ43 | GAA | GAT | CAT | CGA | ATT | GGC | | 801 > 794 |
| | UJ32 | GGT | TTA | TTC | GAC | GTC | TCC | | 1001 > 984 |
| 20 | UJQ | GCT | TTG | AAA | TTG | GCC | AAG | G | 1106 > 1123 |
| | UJ33 | AGA | CTC | CAG | TAT | AAG | AAG | | 1242 > 1225 |
| 25 | UJ26 | AGA | TTT | GCT | GGA | TTT | GAT | AAG G | 1440 > 1461 |
| | UJ34 | ATA | TTT | GAT | GTG | CTC | TCC | | 1664 > 1647 |
| | UJ35 | CTC | AAA | TGA | CTC | CTT | GCT | C | 2058 > 2040 |
| 30 | UJ28 | TGC | GAA | AGA | ACT | ACC | AGG | ATG | 2258 > 2278 |
| | CLFRI-6 | CAG | CTC | TTG | TGA | GTA | GTT | AC | 2527 > 2546 |
| 35 | UJ29 | ATT | CAT | ACT | CTC | CAG | GTC | A | 2662 > 2680 |
| | UJ37R | AAC | AAC | AGT | TAC | CAT | ATG | G | 2767 > 2785 |

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| | | | |
|----|--------------|---------------------------------------|-------------|
| | UJ37 | ACC ATA TGG TAA CTG TTG | 2786 > 2769 |
| | UJ30 | TTA TCC AAT CAA AGG TCT CC | 2835 > 2854 |
| 5 | CLFRI-13 | GTC ATT TAT TTA ACT CCC AA | 2932 > 2951 |
| | CLFRI-13R-RI | CGC GAATTC TTG GGA GTT AAA TAA ATG AC | 2951 > 2932 |
| | CLFRI-14 | GCT CCT GTA ATT GAC ATT TAA G | 3003 > 3024 |
| 10 | CLFRI-15 | CAC TAT CTA AAT AGA CCT C | 3077 > 3095 |
| | UJ44 | TGC GGA TTC CAA CCT TG | 3171 > 3187 |
| 15 | CLFRI-12 | GAT TGT CAA GCT CAA GTT GG | 3298 > 3279 |
| | UJ38 | CAA GAT CAA AGA CTG CTA AAT C | 3360 > 3339 |
| | CLFRI-16 | GTG AGT GTA TCT AGT GTT CA | 3391 > 3372 |
| 20 | UJ39 | CAG AAG CCT CCG GCG AAC | 3761 > 3744 |

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GENERAL METHODS

Growth conditions and measurement of flowering time

The majority of the flowering time analyses were undertaken in a greenhouse under UK summer conditions. Some plants were also grown under defined conditions in Sanyo Gallenkamp Controlled Environment rooms at 20°C. The 16h photoperiod comprised of 10 hours of 400 Watt metal halide power star lamps supplemented with 100 watt tungsten halide lamps and 6 hours using only the tungsten halide lamps. The combination of lamps used for the 10 hour period provided a PAR of 92.9 $\mu\text{moles photons m}^{-2} \text{ s}^{-1}$ and a red:far red ratio of 1.49. The 8 hour extension produced PAR of 14.27 $\mu\text{moles m}^{-2} \text{ s}^{-1}$ and a red:far-red ratio of 0.66.

Flowering time was measured by counting the total number of leaves, excluding the cotyledons. The close correlation between leaf number and flowering time was previously demonstrated for Landsberg erecta and *fca* alleles (Koornneef et al., 1991).

Cosmid and RFLP markers.

DNA of pUC clones mi204, mi51, mi122 were obtained from Bob Whittier and used according to (Lui et al., 1996). Cosmids g8802 and g3843 were obtained from Howard Goodman (MGH, Boston), cultured in the presence of 50 mg/l kanamycin, and maintained as glycerol stocks at - 70°C. CC27P11 was isolated as a random cosmid clone carrying Columbia genomic DNA and mapped by hybridization to yeast artificial chromosome clones (Schmidt et al., 1996). Total cosmid DNA was used as radiolabelled probe to YAC and BAC library colony filters and plant genomic DNA blots.

DNA extractions

Arabidopsis DNA was performed by a CTAB extraction method described by (Dean et al., 1992).

RFLP analysis.

Two to three micrograms of plant genomic DNA was prepared from the parental plants used in the crosses and cleaved in a 300 µl volume. The digested DNA was ethanol precipitated and separated on 0.7% agarose gels and blotted onto Hybond-N filters. Gel transfer to Hybond-N, hybridisation and washing conditions were according to the manufacturer's instructions, except that DNA was fixed to the filters by UV Stratalinker treatment and/or baked at 80°C for 2 h. Radiolabelled DNA was prepared by random hexamer labelling. Radiolabelled probe DNA was hybridised to the filters to identify RFLPs.

RNA extractions

RNA was extracted using a method described by (Dean et al., 1985). polyA RNA was isolated using the polyAtract^R mRNA isolation system (Promega).

RT-PCR analysis

Total RNA was isolated from whole seedlings at the 2-3 leaf stage growing under long days in the greenhouse. For first strand cDNA synthesis, 3 µg of RNA in a volume of 12 µl was heated to 70°C for 3 minutes, and then quickly cooled on ice. 7.5 µl of reaction mix was made containing 1 µl of RNasin, 1 µl of primer (20µM), 4µl of 5x reverse transcriptase buffer (250mM TrisHCl pH8.3, 375mM KCl, 15mM MgCl₂), 2µl DTT (100mM), 1µl dNTP (10mM), 1µl reverse transcriptase (200 units, M-MLV Gibco). This reaction mix was then added to the RNA creating a final volume of 20µl. The mixture was incubated at 37°C for 2 hours and then diluted to 100 µl with water.

5µl of the diluted first strand synthesis reaction was added to 95µl of PCR mix containing 16µl 1.25mM dNTP, 10µl 10xPCR buffer (Boehringer plus Mg), 5µl of a 20mM solution of each of the primers, 63.6µl of water and 0.4µl of 5 units/µl Taq polymerase (Boehringer or Cetus Amplitaq). The reaction was performed at 94°C for 1 minute, 34 cycles of 55°C for 1 minute, 72°C for 2 minutes and then finally at 72°C for 10

minutes.

DNA sequencing

DNA was sequenced using cycle sequencing with Big Dye
5 terminators (PE Applied Biosystems) and then run on an ABI
377 sequencing machine.

Preparation of H51 cosmid library

10 Plant DNA preparation. 30g of H51 plant tissue (in samples of
3g) was ground to a fine powder with liquid nitrogen using a
pestle and mortar. The powder was transferred to a 50ml
centrifuge tube and 20ml of urea extraction buffer [Urea
15 extraction buffer; 8.0M Urea (reagent grade), 0.35M NaCl,
0.05M Tris pH7.5, 0.02M EDTA, 2% sarcosine, 5% phenol (added
after autoclaving) was added]. The tubes were inverted gently
about 30 times and 0.8ml of 20% SDS was added. The tubes
were incubated at 65°C for 10 minutes. 20ml of
phenol/chloroform/IAA was added and the tubes inverted 30
20 times as before and then spun at 3,000 rpm for 20 minutes.
The phenol/chloroform/IAA extraction was repeated and the
aqueous phase removed to 4ml of 5M potassium acetate, 16ml of
isopropanol was added and gently mixed. The nucleic acid was
pelleted immediately by centrifugation at 3000rpm for 20
25 minutes. The resulting pellet was dissolved in 4.1ml of TE.
4.0g of caesium chloride and 0.1ml of 5mg/ml of ethidium
bromide was added. The resulting liquid was transferred to
an ultracentrifuge tube and spun overnight in a vertical
rotor at 53,000rpm. The genomic DNA was removed using a
30 syringe and wide-bore needle into a 15ml Falcon tube.

An equal volume of butanol saturated with sodium
chloride was added to the sample and mixed gently, the
aqueous phase was removed to a clean tube and the process
repeated until the aqueous phase appeared colourless.

35 The caesium chloride was removed by dialysing the sample
against 2l of TE for 4 hours and then overnight in 2l of TE.

The clean DNA was precipitated in a 30 ml Corex tube

with 0.1 volume of 3M Sodium acetate and 2 volumes of 100% ethanol overnight at 4°C. The Corex tube was spun for 10 minutes at 3000rpm, the ethanol poured off and the pellet dried under vacuum, it was re-dissolved in 500ul of TE
5 overnight at 4°C. The OD₂₆₀ of the sample was then measured to ascertain the amount of DNA.

Preparation of pure 04541 vector DNA 50ml of LB + tetracycline (10mg/ml) was inoculated with a single colony of
10 *E.coli* carrying the 04541 vector (Bancroft et al., 1997). This was grown overnight at 37°C and plasmid DNA isolated using the procedure described in (Birnboim and Doly, 1979). The DNA was purified using caesium chloride as described above.

Partial digestion of genomic DNA A small-scale test partial was performed on 10mg of genomic DNA using the restriction endonuclease *Sau* 3A. This was done by using different
15 amounts of the enzyme from 1 unit of enzyme/ug DNA in a reaction mix to 0.08 units of enzyme/mg DNA in a reaction
20 mix. The digest mixes were incubated for 1 hour at 37°C, the reactions were stopped by the addition of 1ml of 0.5M EDTA. The digests were run on an 0.4% agarose gel overnight with DNA size markers which have fragments between 9kb and 30kb.
25 The gel was stained with ethidium bromide solution and a polaroid photograph was taken. The lane (and therefore the most suitable enzyme concentration) containing the most amount of DNA of the required size (between 15kb and 25kb) was noted.

30 Having carried out the small scale test, the rest of the genomic DNA was used in a large scale digestion. The conditions were exactly the same as before except half the enzyme concentration as determined in the small scale test was used. This gave the maximum amount of fragments of the
35 required size. After incubation, 1mg aliquots were removed from the reaction mixes and run on a 0.4% agarose gel overnight to confirm that there were fragments of the

expected size. The rest of the partially digested DNA was precipitated with 0.1 volumes of sodium acetate and 2 volumes of 100% ethanol. The DNA precipitate was rinsed in 70% ethanol and re-dissolve in 270ml of water.

5

Treatment with calf intestinal alkaline phosphatase (CIP) 30ml of CIP buffer and 2.5 units of CIP were added to the 270ml of the partially digested genomic DNA. The mix was incubated at 37°C for 20 minutes and then at 68°C for 15 minutes. 30ml of sodium acetate and an equal volume of phenol/chloroform/IAA were added, mixed and spun for 5 minutes at 13,000rpm. The aqueous phase was removed to 2 volumes of ethanol and precipitated as above. The partially digested, CIP treated DNA was rinsed in 70% ethanol, dried and re-dissolved in 500ml of TE.

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Size fractionation of the genomic DNA A sucrose gradient was prepared with 10-40% sucrose solutions. The DNA in TE was loaded onto the top of the gradient and it was centrifuged for 18 hours at 20,000rpm in a SW41 rotor. 20 fractions of 300-500ml were collected from the bottom of the tube and 10ul of each fraction was run overnight on an 0.4% agarose gel.

20

Purification of fractions of the right size. The fractions from the sucrose gradient which contained partially digested DNA of between 15kb and 20kb were diluted 1 in 4 with water. The DNA was precipitated by the addition of 1/30 volume of 3M sodium acetate pH5.5, 1ul tRNA, and 0.7 volumes of isopropanol and incubation on ice for 60 minutes. The precipitate was pelleted by centrifugation for 20 minutes at 13,000rpm, it was rinsed in 70% ethanol and dried under vacuum. The fractions were redissolved in 5ul of water and then pooled into one tube.

25
30

Preparation of vector DNA 10mg of 04541 was digested with BamH1. An aliquot of 100ng was removed and run on an 0.5% agarose gel with an aliquot of uncut vector to confirm that

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the vector had been digested completely. The rest of the digest was extracted with an equal volume of phenol/chloroform/IAA and precipitated with 0.1 volumes of sodium acetate and 2 volumes of 100% ethanol washed in 70% ethanol and dried under vacuum.

Ligation of the digested vector and the purified fractions

1mg of purified insert DNA was combined with 0.25mg of digested vector DNA in a 7.5ml volume. 0.5ml was removed and stored at -20°C (unligated control). The remaining reaction was incubated at 65°C for 5 minutes, then for 20 minutes at 42°C and finally for 2 hours at room temperature. 1ml of Tris-HCl/MgCl₂, 0.5ml of 0.1M ATP, 0.5ml of 5:1000 dilution of β-mercaptoethanol and 1.0ml of T4 DNA ligase were added and the mix was incubated for 18 hours at 12°C. The unligated control and 0.5ml of ligation were run on a 0.5% minigel to check ligation.

Preparation of plating cells SURETMTetS (Stratagene) were streaked out on L agar plate and grown overnight at 37°C. A single colony was used to inoculate 50ml of LB maltose plus 0.5ml 1M MgSO₄. This was shaken for 4-6 hours at 37°C until the OD₆₀₀ was between 0.5 and 1.0. The bacteria were pelleted at 2,000rpm for 10 minutes and resuspended in 20ml of sterile 10mM MgSO₄. The OD₆₀₀ was measured and more 10mM MgSO₄ was added until the OD₆₀₀ reached 0.5. The cells were stored at 4°C.

Packaging of ligation The packaging into the phage was performed using Gigapack II XL packaging extracts from Stratagene and the protocol accompanying the extracts was followed. 10ml of phage stock was added to 100ml of phage buffer and 200ml of plating cells and incubated at 37°C for 20 minutes. 1ml of L broth was added and incubation continued for another hour. 0.1ml and 0.5ml aliquots were spread on a L agar tet10mg/ml plate and incubated overnight. Colonies were counted and the titre was calculated.

Transformation of Arabidopsis

The cosmids containing DNA from the vicinity of *FRI* were mobilised into *Agrobacterium tumefaciens* C58C1 using triparental mating (Hoekema et al., 1983). The T-DNA was introduced into *Arabidopsis* plants using the vacuum-infiltration technique as described in (Bechtold et al., 1993).

10 *References*

Bancroft et al. (1997). *Weeds World* 4ii,

Bechtold, N., Ellis, J. and Pelletier, G. (1993). *In planta*
15 *Agrobacterium* mediated gene transfer by infiltration of adult
Arabidopsis thaliana plants. *C.R.Acad.Sci.Paris* 316,1194-1199.

Birnboim, H.C. and Doly, J. (1979). *Nucleic Acids Res.*
20 7(6),1513-1523.

Burn, J.E., Smyth, D.R., Peacock, W.J. and Dennis, E.S.
(1993). Genes conferring late flowering in *Arabidopsis thaliana*. *Genetica* 90,145-157.

25 Clarke, J.H. and Dean, C. (1994). Mapping *FRI*, a locus
controlling flowering time and vernalization response in
Arabidopsis thaliana. *Mol.Gen.Genet.* 242,81-89.

30 Cohen, C. and Parry, D.A.D. (1986). α -helical coiled coils -
a widespread motif in proteins. *TIBS* 11,245-248.

Dean et al. (1985). *Embo J.* 4,3055-3061.

35 Dean et al (1992). *Plant J.* 2,69-81.

Hoekema et al (1983). *Nature.* 303,179-180.

Koornneef et al (1991). Mol.Gen.Genet. 299,57-66.

5 Koornneef, M., Blankestijn-de Vries, H., Hanhart, C., Soppe, W. and Peeters, T. (1994). The phenotype of some late-flowering mutants is enhanced by a locus on chromosome 5 that is not effective in the Landsberg erecta wild-type. Plant J. 6, 911-919.

10 Koornneef, M., Alonso-Blanco, C., Peeters, A.J.M. and Soppe, W. (1998). Genetic control of flowering time in Arabidopsis. Annu.Rev.Pl.Phys.Pl.Mol.Biol. 49, 345-370.

15 Lee, I., Bleecker, A. and Amasino, R. (1993). Analysis of naturally occurring late flowering in *Arabidopsis thaliana*. Mol. Gen. Genet. 237, 171-176.

20 Lee, I., Michaels, S.D., Masshardt, A.S. and Amasino, R.M. (1994). The late-flowering phenotype of *FRIGIDA* and mutations in *LUMINIDEPENDENS* is suppressed in the Landsberg erecta strain of *Arabidopsis*. Plant J. 6, 903-909.

25 Lui, Y-G., Mitsukawa, N., Lister, C., Dean, C. and Whittier, R.F. (1996). Isolation and mapping of a new set of 129 RFLP markers in *Arabidopsis thaliana* using recombinant inbred lines. Plant J. 10, 733-736

Lupas, A., van Dyke, M. and Stock, J. (1991). Predicting coiled coils from protein sequences. Science 252, 1162-1164.

30 Napp-Zinn, K. (1957). Untersuchungen zur genetik des kaltebedürfnisses bei *Arabidopsis thaliana*. Zeitschrift für induktive Abstammungs- und Vererbungslehre 88, 253-258.

35 Napp-Zinn, K. (1962). über die genetischen Grundlagen des Vernalisationsbedürfnisses bei *Arabidopsis thaliana*. I. Die Zahl der beteiligten Faktoren. Z.Vererbungslehre 93, 154-163.

Napp-Zinn, K. Theory of vernalization - new experiments with *Arabidopsis*. In: *1st International Symposium on Arabidopsis Research*, Göttingen: Supplement, Arabidopsis Information Service, 1965, p. 56-61.

5

Napp-Zinn, K. Vernalization - environment and genetic regulation. In: *Manipulation of Flowering*, edited by Atherton, J.G. London: Butterworths, 1987, p. 123-132.

- 10 Osborn, T.C., Klee, C., Parkin, I.A.P., Sharpe, A.G., Lydiate, D.J. and Trick, M. (1997). Comparison of flowering time in *Brassica rapa*, *B. napus* and *Arabidopsis thaliana*. *Genetics* 146, 1123-1129.
- 15 Sanda, S.L. and Amasino, R.M. (1996). Interaction of *FLC* and late-flowering mutations in *Arabidopsis thaliana*. *Mol.Gen.Genet.* 251, 69-74.
- 20 Schmidt, R., West, J., Love, K., et al. (1995). Physical map and organization of *Arabidopsis thaliana* chromosome 4. *Science* 270, 480-483.
- Schmidt et al (1996). *Plant J.* 9, 755-765.

25 SEQUENCES OF VRN2

Landsberg erecta VRN2 cDNA (SEQ ID NO: 57)

CAAGCTTCTTCAATTTTGCTTGCTCTCTCTTACACAGCAATCGGTGTTTTTCGCAGCTTTCA
GGCCTCAATCCAAGACATTCTATATAAGCATATTGCAGAAGAGGCGGTTCTAATTGTTGCAT
TGAGTTTATCGCTATGACGTAGGGAAATTCTAATTAGGGGAGGCCTCAGAGTTTGCCTAA
CTTCATAATCGGCTCTTGACGTTGTTGAGTGTATTGAACAAGAATGTGTAGGCAGAATTGT
CGCGCGAAATCCTCACCGGAGGAAGTGATTTAACTGATGAGAATCTCTTGATATATTGTAA
ACCTGTTGACTATATAACATCTTTCACCTTCGCTCTCTAGGCAACCCATCGTTTCTTCCAA
35 GATGCTTGAAGTACAAAATTGGAGCAAAGCGCAAAGAAAGTCAAGATCTACTGGGATGGTA
GTTTTCAACTATAAGGATTGTAATAACACATTACAGAAACTGAAGTTAGGGAGGATTGTTT
TTGTCCATTTTGCTCTATGCTATGTTGGTAGCTTCAAGGGGCTGCAATTTCAATTGAATTCAT

CTCATGATTTATTTGAATTTGAGTTCAAGCTTTTCGAAGAATACCAGACAGTTAATGTTTCT
 GTAAACTTAATTCCTTCATATTTGAGGAAGAAGGAAGTGATGACGATAAATTTGAGCCCTT
 CTCTCTCTGCTCGAAACCTCGTAAGCGGAGACAAAGAGGTGGCAGAAATAACACCAGGAGAC
 TTAAAGTATGCTTTTTTACCGTTGGATTACCCAGTTTAACTAATGGCACAGAAAATGGAATC
 5 ACCCTACTTAATGATGGAAACCGTGGTTTAGGATATCCCGAGGCAACAGAGCTTGCTGGACA
 ATTTGAGATGACCAGCAACATTCCACCAGCCATAGCCCCTCTTCTCTGGACGCTGGTGCTA
 AAGTTATATTGACAAGCGAAGCTGTGGTCCCTGCTACTAAGACAAGAAAGTTATCTGCTGAG
 CGATCAGAGGCTAGAAGCCACCTACTTCTTCAGAAACGCCAATTCTATCATTCTCACAGAGT
 CCAGCCAATGGCGCTTGAGCAAGTAATGTCTGACCGGGATAGCGAGGATGAAGTCGATGACG
 10 ATGTTGCAGATTTTGAAGATCGCCAGATGCTTGATGACTTTGTGGATGTGAATAAAGATGAA
 AAGCAATTCATGCATCTTTGGAACTCGTTTGTAAGAAAACAAAGGGTTATAGCAGATGGTCA
 TATCTCTTGGGCATGTGAAGCATTTTCAAGATTTTACGAGAAAGAGTTGCACCGTTACTCAT
 CACTCTTCTGGTGTGGAGATTGTTTTTGATTAACTATGGAACCATGGACTTGTGACTCA
 GCCACCATCAACAACGCAATACCATCCTCGAGAATTGCCGTAATAGCTCAGACACCACCAC
 15 CACCAACAACAACAACAGTGTGGATCGTCCCAGTGACTCAAACACCAACAACAATAACATTG
 TGGATCATCCCAATGACATAAACAACAAGAACAATGTTGACAACAAGGACAATAACAGCAGA
 GACAAAGTAATTAAATAGGAAAATCTCCGGCTTTTATGATACCGATTATCGGATTGTAAC
 TATCTCTCTTCTTAAAAAATTGTTTAGGAGCAAACAAATTTTTTATATGTTAGTGTATTCA
 ACTGATTACATTTTTTAGTTAAAAAATAATGGATTCTGCTTATAACT

20 Columbia VRN2 cDNA (SEQ ID NO: 58)

CAAGCTTCTTCAATTTTGCTTGCTCTCTCTTACACGGCCAATCGGTGTTTTTCGCAGCTTT
 CAGGCCTCAATACAAGACATTCTATATAAGCATATTGCAGAAGAGGCGGTTCTAATTGTTGC
 25 ATGGAGTTGAACAATATGACGTAGGGAAATTCTAATTTAGGGGAGGCCTCAGAGTTTGCCT
 AACTTCATAATCAGCTCTGGACGTTGTTGATTGTATTTGAACAAGAATGTGTAGGCAGAATT
 GTCGCGCGAAATCCTCACCGGAGGAAGTGATTTCAACTGATGAGAATCTCTTGATATATTGT
 AAACCTGTTGACTATATAACATCTTTCACCTTCGCTCTCTAGGCAACCCATCGTTTCTGCC
 AAGATGCTTGAACACAAAATTGGGGCAAAGCGCAAAGAAAGTCAAGATCTACTGGGATGG
 30 TAGTTTTCAACTATAAGGATTGTAATAATACATTACAAAGAACTGAAGTTAGGGAGGATTGT
 TCTTGTCCATTTTGCTCTATGCTATGTGGTAGCTTCAAGGGGCTGCAATTTTCATTTGAATTC
 ATCTCATGATTTATTTGAATTTGAGTTCAAGCTTTTGGAGAATAACCAGACAGTTAATGTTT
 CTGTAAACTTAATTCCTTCATATTTGAGGAAGAAGGAAGTGATGATGATAAATTTGAGCCC
 TTCTCTCTCTGCTCGAAACCTCGTAAGCGTAGACAAAGAGGTGGCAGAAATAACACCAGGAG
 35 ACTTAAAGTATGCTTTTTTACCGTTGGATTACCCAGTTTAGCTAATGGCACAGAAAATGGAA
 TTGCCCTGCTGAATGATGGAAACCGTGGTTTAGGATATCCCGAGGCAACAGAGCTTGCTGGA
 CAATTTGAGATGACTAGCAACATTCCACCAGCCATAGCCCCTCTTCTCTGGACGCTGGTGC

